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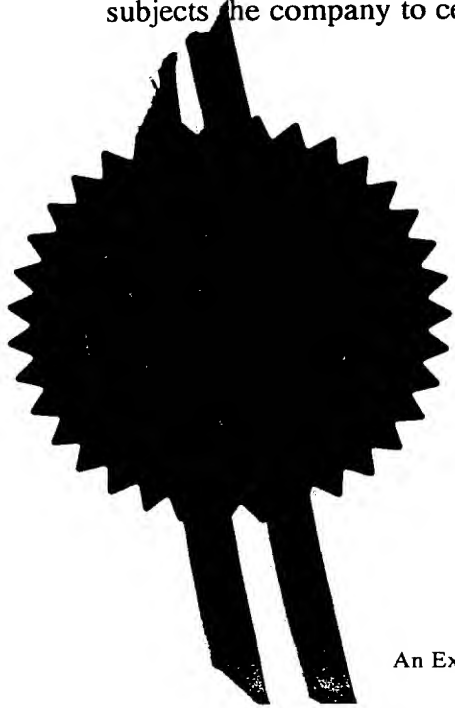
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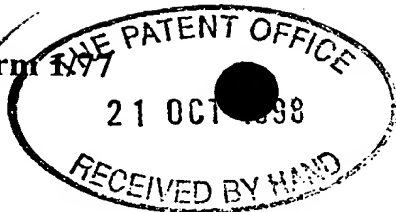
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1.	Your reference	27.59.68443

2.	Patent application number *(The Patent Office will fill in this part)*	2 1 OCT 1998 **9823071.7**

3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Affibody Technology AB c/o Pilotgatan 22 S-128 32 Skarpnäck Sweden
	Patents ADP number *(if you know it)*	7536766001
	If the applicant is a corporate body, give country/state of incorporation	Sweden
4.	Title of the invention	A Method
5.	Name of your agent *(if you have one)*	Frank B. Dehn & Co.
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	179 Queen Victoria Street London EC4V 4EL
	Patents ADP number *(if you know it)*	166001

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68443.602

A Method

The present invention relates to affinity separation and to ligands for use therein.

Affinity separations generally take place on an affinity chromatography column and take advantage of the potentially highly specific nature of interactions involving biomolecules, especially proteins. Other possible separation methods include membrane filtration, two-phase extraction, fluidised beds, expanded beds and magnetic bead separation (Scopes, R.K. Protein Purification, Principles and Practice, 3rd Ed. ISBN 0-387-94072-3). Interactions include protein-protein, protein-nucleic acid, enzyme-substrate, receptor-ligand (e.g. hormone) protein- carbohydrate and protein-metal interactions. Generally, affinity separation is based on the biologically important binding interactions that occur on protein surfaces, such as that between an enzyme and its substrate, between nucleic acid and a DNA binding protein or between antigen and antibody. Either one of a pair of binding partners can be immobilised, e.g. by covalent bonding to an insoluble matrix in an affinity column in order to assay for or purify its binding partner. The very selective nature of interactions between or involving proteins makes them ideal for purification/separation techniques and for many applications, affinity chromatography involving an immobilised protein ligand is preferred over ion-exchange or gel-filtration chromatography.

If a sample is added to a column which carries immobilised on a solid matrix a specific binding partner to a target molecule in the sample, the target protein will be retained in the column while the majority of the non-target molecules will simply run through. A solution, typically containing a gradually decreasing pH can be added to the column to wash through firstly any

non-specifically bound molecules and finally to elute the target molecule. The target molecule, having the highest affinity for the immobilised ligand, will be the last molecule to be washed off the column and it is therefore the final fractions which will contain the highest concentration of the target protein and can then be used in a further purification/concentration step or be assayed directly or indirectly for the presence of the target molecule.

Medicine and research in the biochemical and biotechnological fields generally, has created a demand for ever purer samples of organic and biological molecules and thus for strategies which can provide samples of high purity as quickly and cheaply as possible.

As well as a need to obtain pure samples of proteins and other molecules, affinity separation is important in assaying samples quantitatively and qualitatively for a target molecule. This may be important, for example when blood or urine samples are being assayed for molecules indicative of a disease such as a metabolic disorder or even the presence of non-naturally occurring substances, narcotics, steroid derivatives etc.

An affinity chromatography column, complete with its solid affinity matrix, can be expensive and clearly it is desirable to be able to re-use the column several times, i.e., to complete a number of runs before it has to be discarded due to reduced ability of the immobilised ligand to bind a target molecule or to a reduced specificity of binding. In order to achieve a set of results which can be directly compared and to clear the column of non-specific and specifically bound molecules before performing another run, it is necessary to clean the column. The recognised standard for cleaning and sanitizing separation media and systems is NaOH, often in combination with NaCl. An applied 0.1-

1.0 M NaOH solution is able to remove viruses, bacteria, nucleic acid, proteins, yeasts, endotoxins, prions and other contaminating agents. The NaOH contact time may vary, between 30 minutes and 1 hour is typical, and removal from the system is monitored by simple in-line pH and conductivity measurements.

However, the ability of the separation media to withstand these rather harsh sanitizing conditions depends on the functional groups of the attached ligand(binding partner), attachment chemistries, and the stability of the base matrices to alkaline conditions. Proteins are sensitive to extreme pH, such as is experienced during NaOH cleaning and, generally speaking, this will adversely affect the effectiveness of protein-based affinity media. Thus, although protein based affinity separation has advantages over ion exchange and gel-filtration chromatography due to its good specificity, these other less specific techniques are not adversely affected by standard cleaning methods.

The sensitivity of proteins to alkaline pH is primarily due to deamidation of asparagine and glutamine residues, particularly asparagine residues. Deamidation of asparagine results in the formation, via a cyclic imide intermediate, of isoaspartate and aspartate, usually in the ratio of 3:1 to 4:1. This reaction does take place at physiological pH but is far faster at alkaline pH such as present in a chromatography column which is being cleaned by an NaOH solution. The isoaspartyl form is characterised by an atypical amide bond between the β-carboxyl of aspartate and the α-nitrogen of the C-flanking amino acid. This results in an extra $-CH_2-$ in the backbone of the protein as well as a free α-carboxyl group. Cleavage of the peptide backbone may occur as a result of the deamidation and the protein may lose its activity due to a structural change in the whole protein, or merely a small change in a sensitive region such as the active or binding site.

The susceptibility of asparagine residues to deamidation
is sequence and conformation dependent, Asn residues at
Asn-Gly and Asn-Ser sites being particularly vulnerable.

It is a serious problem when an affinity
chromatography column has been set up with a protein
immobilised on an insoluble support within it and the
necessary cleaning process between runs results in
reduced efficacy of the system. As well as hastening
the end of the absolute useful life of the column, if
successive washes decrease the ability of the
immobilised protein to capture its binding partner from
a sample, comparisons between runs where the
concentration of an analyte in a series of samples is
measured become meaningless. There is therefore a need
for a method of affinity separation wherein the
immobilised protein is less susceptible to standard
cleaning methods, particularly to alkaline pH.

Thus, in one aspect, the present invention provides
a method of affinity separation wherein the affinity
ligand is an immobilised proteinaceous ligand wherein
one or more of its asparagine residues has been
modified.

The term modified includes deletion of the
asparagine residue or replacement of it with a less
alkaline sensitive amino acid or wherein the asparagine
residue has been modified by substitution or other
chemical derivitisation, e.g. by a protecting group.
Replacement of one or more asparagine residues with a
less alkaline sensitive amino acid is preferred.

By 'affinity separation' is meant any purification
or assaying technique which involves the addition of a
sample containing a target analyte to a solid which
carries on it a specific binding partner to the analyte.
Gravity or other means allows the sample to pass through
or across the solid, and the interaction between the
analyte and its specific binding partner immobilised on
the solid means that the analyte will be retained on the

solid while the rest, or most of the rest, of the sample passes through the system. The separation may conveniently be carried out on an affinity chromatography column. The solid is preferably arranged in a column, so that the sample can be added to the top and the non-target part of the sample runs off. An eluant such as a salt solution or a change of pH can be used to displace the specifically bound analyte which can then be collected in a number of aliquots in a controlled manner.

'Analyte' is used to refer to any molecule or fragment of a molecule, proteinaceous or otherwise in a sample which is capable of binding specifically to the immobilised ligand.

The term 'specific binding partner' may include a molecule or a group of related molecules and any one of these molecules may also bind 'specifically' to one or more other molecules. Thus, the term does not imply that any one immobilised ligand or analyte can have only one binding partner, rather that binding is specific to the extent that most other molecules will not bind with the same affinity or with the same stringency, particularly that other non-target molecules in a given sample will have a much lower affinity. Purification by affinity separation is often of the order of several thousand-fold because of the high affinity between biologically specific binding partners.

All types of affinity separation which include an immobilised proteinaceous ligand can be used in the method of the invention, and suitable matrices known for use with particular ligands are known in the art, typically based on chromatography using agarose, polacrylamide, silica, polyvinyl styrene, dextran or other polymers. The protein ligands are usually attached to the matrix by a coupling agent such as cyanogen bromide, epichlorohydrin, bisoxirane, divinyl sulfone, carbonyl diimidazol, N-hydroxysuccinimide,

tosyl/tresyl chloride, epichlorohydrine, carbodiimide, glutaraldehyde, hydrazine, oxirane and also carboxyl or thiol activated matrices and again such coupling agents and coupling chemistries are well known in the art and widely described in the literature (Jansson, J.C. and Rydén, L. Protein purification, 2nd Ed. pp 375-442, ISBN 0-471-18626-0). Various derivatives of matrices which allow straightforward immobilization include CNBr-activated Sepharose 4B, AH-Sepharose 4B and CH-Sepharose 4B and Epoxy-activated Sepharose 6B (Pharmacia).

The proteinaceous affinity ligand may be a molecule having a protein component, which may function as a "specific binding partner" as defined above. Thus, glycoproteins or protein-lipid complexes or indeed proteins with prosthetic groups may be used as the affinity ligand which is modified according to the present invention. Protein molecules are, however, preferred.

Suitable amino acids to replace asparagine include any of the other 19 standard naturally occurring amino acids, although cysteine and glutamine would not be preferred. Preferred substituting amino acids include lysine, aspartic acid and leucine. Non-naturally occurring amino acids and amino acid derivatives which are well known to the man skilled in the art could also be used to replace asparagine residues. Significant improvements in the stability of the immobilised protein and thus the efficacy of the column after cleaning can be observed when just one asparagine is substituted by a less alkaline sensitive residue but preferably 2, 3 or more, or even all of the asparagine residues are substituted.

As indicated above, the sensitivity of a particular asparagine residue to deamidation will depend on the configuration of the protein, it being particularly important to replace those residues which are on the surface of the three dimensional structure of the

protein and thus particularly exposed to the alkaline
conditions. It is the overall stability of the ligand
which is of most importance and it is desirable to
retain the specificity of the interaction between the
immobilised ligand and its binding partner, therefore,
preferably it is the asparagine residues not involved in
the ligand-analyte interaction which are replaced.

The "asparagine modified" proteinaceous affinity
ligand (hereinafter the "modified protein") may be made
by any method known to the skilled man. Standard
techniques for site-directed mutagenesis of nucleic acid
are described, for example, in the laboratory manual
entitled Molecular Cloning by Sambrook, Fritsch and
Maniatis. A preferred technique involves PCR
mutagenesis, where primers which incorporate the
necessary mis-match base pairs to generate the desired
mutations are used in a first round of PCR. In the
second run, the fragments from the first run are mixed
and the polymerase is able to fill in the strands. The
resulting double stranded fragment can be ligated into a
plasmid which is then used to transform *E. coli*. The
protein can by synthesised in vitro without using a
biological host and in this case non-naturally occurring
amino acids can be introduced.

As well as the many general uses of affinity
separation indicated previously, of particular interest
in the present case is the use of affinity separation
with ligands which have been made by
randomisation(random mutagenesis) of a particular
protein to generate ligands with novel, modified or
enhanced binding characteristics. These proteins are
referred to as combinatorial proteins. Such a technique
typically involves random mutagenesis of a target
protein, expression of the full library of these
variants, e.g. on the surface of filamentous
bacteriophage, followed by selection of a protein
exhibiting the desired binding characteristics, this

selection typically involving a binding reaction between the variant protein and an immobilised ligand (binding partner). The mutagenesis is random in that the resulting amino acid encoded by any particular codon is not generally pre-determined but the positions where mutations are to be introduced are generally identified in advance.

The use of an expression system such as surface display on phage provides a crucial link between genotype and phenotype; there is a self-contained unit which can be selected on the basis of its specific binding interactions and which also carries the nucleic acid encoding for the protein responsible for the observed binding characteristics. This enables expression in useful amounts of the protein selected for its binding characteristics, such expression typically taking place in a transformed bacterial host.

The protein, selected by its ability to bind to an immobilised ligand, is then itself used in affinity separation (i.e. as the affinity-ligand). It is immobilised and used to purify or assay for a target molecule in a sample, typically the same ligand which was used to select the protein in the first place. In this way, a protein from a library of variants can be selected for its ability to bind e.g. insulin, using a column with insulin immobilised on a matrix therein and the selected protein can then be used to test samples for the presence of insulin.

If a protein is stabilised by replacing one or more of its asparagine residues with a less alkaline sensitive amino acid prior to the randomisation and selection steps described above, then its useful life as an affinity ligand will be extended due to its ability to withstand the harsh conditions such as high pH which are experienced when an affinity column is washed between runs.

Therefore, a further aspect of the present

invention comprises a combinatorial protein wherein in a step separate from the randomisation step, the stability of the protein in alkaline conditions has been increased by modifying one or more of its asparagine residues (preferably by replacing one or more of its asparagine residues with a less alkaline sensitive amino acid).

Nucleic acid molecules encoding such a protein as well as cells expressing the protein constitute further aspects of the present invention.

The randomisation may itself result in substitution of asparagine residues but this aspect of the invention is concerned with proteins which have also been specifically modified to replace one or more asparagine residues in order to increase stability. The protein can be stabilised before or after the randomisation or at the same time but preferably the stabilising modifications will be introduced before the randomisation takes place. If stabilisation is performed before randomisation, it need only be performed once, and by selecting from the library of randomised variants against a number of ligands, several stabilised proteins with different binding characteristics can be obtained. If the stabilisation is performed after selection for a particular binding affinity, then there is a risk that some affinity would be lost as a result of the stabilising substitutions.

Techniques for construction of a combinatorial library of protein molecules and subsequent selection to obtain proteinaceous ligands having desired binding characteristics are known in the art (Nygren, P. and Uhlén, M. Current Opinion in Structural Biology (1997) 7: 463-469). Generally, a protein molecule perhaps having intrinsic beneficial properties such as temperature or pH insensitivity is used as a scaffold and a combinatorial library is then constructed via random but targeted amino acid substitutions of that protein molecule, in order to produce a library of

molecules having different binding characteristics. Surface residues are generally targeted for random mutagenesis.

Suitable protein scaffolds may simply be linear peptides but preferably the scaffold will possess a folded three dimensional structure which has the potential for higher affinities and is less susceptible to proteolytic degradation. Rather than designing a scaffold *de novo*, naturally existing proteins or domains are usually selected for further engineering. For the avoidance of doubt, it is to be noted that throughout this specification the word protein is used to refer to whole protein molecules as well as domains or fragments thereof, polypeptides or peptides.

The choice of protein scaffold depends on several parameters including an ability to be effectively expressed in *E. coli* when the randomised protein is to be displayed as a fusion protein with a filamentous phage coat protein. The protein should also comprise sufficiently large regions on its surface which are tolerant to substitution (or insertion) without losing the overall three dimensional structure. If the library is to be produced synthetically, a small overall size is a prerequisite. Where the selected scaffold protein has a binding function, amino acid residues involved in that interaction may be a target for randomisation. Randomisation may be performed in order to enhance known binding properties or to develop ligands with new specificities.

Suitable scaffold molecules are discussed in Nygren et al. (1997) and include cyclic peptides having 40 or more residues in a constrained sequence, immunoglobulin-like scaffolds including Fv or single-chain (scFv) domains, bacterial receptors such as the 58-residue one-domain *Staphylococcal* protein A analogue Z, DNA-binding proteins particularly zinc fingers and protease inhibitors. All these molecules can be stabilised by

substituting one or more native asparagine residues with a less alkaline sensitive amino acid before a combinatorial library of the pre-stabilised protein is made.

Of particular interest is the bacterial receptor domain Z (Nord, K., Nilsson, J., Nilsson, B., Uhlén, M. and Nygren, P. Protein Engineering (1995) 8, 6, 601-608). This paper by Nord et al. describes a suitable method of constructing a combinatorial library of protein molecules which can be applied to a range of scaffold molecules. The method described is solid-phase-assisted and based on the stepwise assembly of randomised single-stranded oligonucleotides.

Selection from a generated protein library can be performed in a number of different ways known in the art, including bead immobilised libraries (McBride, J.D., Freeman, N., Domingo, G.J. and Leatherbarrow, R.J., J. Mol. Biol. [1996] 259: 819-827), fusions to DNA-binding proteins (Schatz, P.J., Biotechnology [1993] 11, 1138-1143) and when displayed on bacteria (Lu, Z., Murray, K.S., Van Cleave, V., LaVallie, E.R., Ståhl, M.L. and McCoy, J.M. Biotechnology [1995], 13 366-372) or phage (Clackson, T. and Wells, J. TIBTECH (1994) 12, 173-183) as well as yeast cells,(Boder, E.T. and Wittrup, K.D., Nature Biotechnology (1997) 15, 553-557) and in viral systems (Ernst, W., Grabher, R., Wegner, D., Borth, N., Graussauer, A. and Katinger, H. Nucleic Acids Research (1998) 26, 1718-1723 and Grabher, R., Ernst, W., Doblhoff-Dier, O., Sara, M. and Katinger, H. BioTechniques (1997) 22, 730-735).

International Patent Application, publication No. WO 95/19374 describes the generation of a combinatorial library of Z-variants, see in particular Example 4. This application discusses on page 12 the advantages of the Z domain as an affinity ligand in the purification of recombinant proteins, as it is relatively stable in the harsh environment of an affinity column during

cleaning. The present invention offers further benefits
in that the Z domain can be stabilised to alkaline pH by
replacement of asparagine residues. According to the
present invention it is proposed to stabilise the native
Z domain before the combinatorial library is prepared.

Thus in a further aspect, the present invention
provides a method of preparing a combinatorial library
of protein molecules wherein the protein has been
rendered less sensitive to alkaline pH by modification
of one or more of its asparagine residues before it is
randomised (preferably by replacement of one or more of
its asparagine residues).

In a yet further aspect of the present invention is
provided a method of phage display wherein a protein
expressed on the phage surface has had one or more of
its asparagine residues modified (preferably by
replacement with a less alkaline sensitive residue) in a
step separate to any modifications introduced in order
to modify binding characteristics of the protein.

A further aspect of the present invention comprises
a method of making a stabilised combinatorial protein
comprising the steps of:

a)   modification of asparagine residues within a
protein molecule to increase stability of the protein in
alkaline conditions; and

b)   randomisation of the protein molecule to
modify its binding characteristics.

Modification in step a) will preferably comprise
replacement of asparagine residues with other less
alkaline sensitive amino acids, Step a) is preferably
carried out before step b).

As well as a protein molecule which has been
randomised and stabilised, the present invention also
relates to fusion proteins comprising a stabilised part
and a randomised part, such proteins being useful in
certain applications. In particular, this technique
enables the development of a stable framework which can

have attached thereto a variable region with specific
binding characteristics. The variable region can be
prepared by randomisation techniques as discussed above
and a protein molecule having desired binding
characteristics can be selected from the library of
variants, for example by phage display and affinity
chromatography. This randomised protein can then be
expressed as a fusion protein, e.g. in *E. coli,* together
with a protein molecule which has already been
engineered to improve its stability to alkaline
conditions by replacing one or more of its asparagine
residues with other less alkaline sensitive residues.

The fusion protein can be used as an immobilised
ligand in affinity chromatography, giving the benefits
of a generally stable molecule provided by the framework
part as well as a pre-selected binding affinity provided
by the randomised part. Such a system may enable the
use of a small protein molecule during the randomisation
and selection stage as important protein-like
characteristics (as opposed to peptide characteristics)
are provided by the stabilised part. The same
stabilised part can be used together with a variety of
different randomised molecules and vice versa. Suitable
protein molecules to perform as the stabilised framework
part of the fusion peptides include Albumin Binding
Protein (ABD); the bacterial receptor domain Z is a
suitable molecule for randomisation in this context.

Thus the present invention also provides a fusion
protein comprising a first part wherein one or more
naturally occurring asparagine residues have been
modified (preferably replaced by an amino acid residue
less sensitive to high pH) and a second part being a
randomised protein molecule selected for its specific
binding properties.

Nucleic acid molecules encoding such a protein as
well as cells expressing the protein constitute further
aspects of the present invention.

A further aspect of the present invention comprises a method of stabilising an affinity ligand by modifying one or more of its asparagine residues (preferably the affinity ligand is stabilised by replacing one or more of its asparagine residues with an amino acid residue less sensitive to alkaline pH).

A still further aspect comprises the use of a protein molecule stabilised by modification of one or more of its asparagine residues in surface display or in affinity chromatography.

The term 'surface display' refers to the technique involved in selection of a protein from a library of molecules which are presented (displayed) in a manner which enables differentiation between the protein molecules on the basis of their binding characteristics. Surface display is typically performed on the surface of filamentous bacteriophage (phage display) but display can also be on the surface of bacteria, yeast cells or using viral systems.

A preferred affinity ligand for use in the methods of the invention is Albumin Binding Protein (ABD), a protein domain with affinity towards human serum albumin (HSA). It is derived from a cell wall anchored bacterial receptor protein from *Streptococcus* G148. It is of particular use as an affinity ligand for the purification of human serum albumin (HSA). The wild type sequence of this protein has four asparagine residues, enhanced stability is observed when just one of these residues is substituted but preferably all four residues are replaced by less alkaline sensitive residues. The wild type amino acid sequence of ABD is:
LAEAKVLANRELDKYGV-SDYYKNLINNAKTVEGVKALIDEILAALP

the asparagine residues have been indicated in bold.

Thus a further aspect of the present invention is Albumin Binding Protein(ABD) or fragments or derivatives thereof wherein one or more native asparagine residue

have been replaced by a less alkaline sensitive amino acid. Nucleic acid molecules encoding such a protein as well as cells expressing the protein constitute further aspects of the present invention.

In a particularly preferred embodiment Asn_8 is replaced by leucine, Asn_{22} by aspartic acid, Asn_{25} by aspartic acid and Asn_{26} by lysine. Asparagine is the most alkaline sensitive amino acid and so any other amino acid residue could be used to replace it and an increase in stability would be expected. It may be of assistance to compare the sequences of other homologous proteins, which are found in other species or perform the same role, to identify suitable amino acids to be used in the substitution.

In a method suitable for the stabilisation of other affinity ligands, the modifications were introduced by PCR mutagenesis. Firstly mismatched primers are used to introduce the mutations and then the fragments from the first run of PCR are mixed and the polymerase allowed to fill in the strands. The double stranded fragment of ABD incorporating the modifications is cleaved with restriction enzymes and ligated into a plasmid restricted with the same enzymes, which is then used to transform _E. coli_, the expression system for the protein.

Preferably, the affinity ligand purified according to the methods of the invention retains more than 80%, preferably more than 95%, of its normal binding capacity after 20 rounds of treatment with 0.5M NaOH.

The invention will be further described in the following non-limiting Examples in which:

Figure 1 is a schematic description of the PCR-mutagenesis step according to the invention;

Figure 2 shows the cloning of the gene construct;

Figure 3 is the amino acid sequence of wild type ABD and the mutated alkaline resistant variant of it;

Figure 4 shows an SDS-PAGE (4-20%) under reducing

conditions. Lanes 1 and 2 show purified ABD and ABDstab
respectively. The third lane shows a molecular weight
marker (from top 94, 67, 43, 40, 20.2 and 14.4 kDa
respectively). In order to check the selectivity of the
mutated ABD, E. coli disintegrate was spiked with HSA
(lane 4) and loaded onto the column. Lanes 5, 6 and 7
show the eluted material after 2, 8 and 15 rounds of
NaOH treatment. Lane 8 shows an HSA reference;

Figure 5 - Sensorgrams from the BIACore showing the
binding characteristics of (A)ABDwt and (B)ABDmut before
and after treatment with 0.5M NaOH for 24 hours; and

Figure 6 is a diagram showing the capacity of two
affinity matrices, ABDwt and ABDmut respectively, after
several rounds of treatment with 0.5M NaOH.

Example 1 - Cloning

As a template for the PCR mutagenesis pTrpABDT1T2 was
used (Kraulis et al. (1996) FEBS Lett. 378, 190-194).
The plasmid encodes the gene for ABDwt under control of
the tryptophan promoter. The PCR mutagenesis was
performed in two steps. In the first PCR the mutations
was introduced and in the second round of PCR, the
fragments from the first run were mixed and the
polymerase was allowed to fill in the strands (Figure
1). The primers used in the first PCR were:
5'-ACGTAAAAG GGTATCTAGA ATTATGAAAG C-3' (SOHO8)
3'-CAGAATCGAG ACTCTCTCGA GCTGTTTATA CC-5' (SOHO6)
5'-GAGAGAGCTC GACAAATATG GAGTAAGTGA CTATTACAAG
GATCTAATCG ATAAAGC-3' (SOHO7)
3'-CCGCCTACTC TCTTCTAAAA GTCG-5' (T1T2)

The double stranded fragment was cleaved with Xba I and
Pst I and ligated in to pTrpABDT1T2 restricted with the
same enzymes (Figure 2). The ligation mixture was used
to transform E. coli, strain RRIΔM15. After confirming
the size of the inserts by PCR, the sequence was

verified by cycle sequencing (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting plasmid was denoted pTrpABDmutT1T2.

Example 2 - Expression and purification

E. coli cells harboring the plasmid encoding ABDmut and also the wild type ABD as a reference were grown over night in shake flasks containing 500 ml Tryptic Soy Broth (30 g/l) supplemented with yeast extract (Difco, USA) (5 g/l) and kanamycin monosulphate (50 mg/l). Since the ABD gene is under control of the tryptophan promoter, the m-RNA production starts when the amino acid is missing in the growth medium. Cells were harvested after 20 hours by centrifugation, 4000xg in 10 minutes. After resuspending the cells in 30 ml TST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) they were fractured by sonication. After that a centrifugation step was performed, approximately 40,000xg in 20 minutes. The supernatant was filtered (0.49 μm). The soluble fusion protein was isolated by affinity chromatography on human serum albumin (HSA) Sepharose as described by Stahl *et al.* (1989) J. Immunol. Meth. **124**, 43-52. The protein content in eluted fractions was measured by absorbance at 280 nm and relevant fractions were collected and lyophilized. Both proteins are of high purity after a single step purification and migrate in accordance with their molecular masses.

Example 3 - Binding characteristics before and after NaOH-treatment

In order to examine the stability of ABDwt and ABDmut, the binding characteristics were analysed before and after treatment with 0.5 M NaOH in room temperature for 24 hours. Both ABDwt and ABDmut were solubilized in

1xHBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20) to a concentration of 800 nM and analyzed on BIAcore 2000 (Biacore AB, Uppsala, Sweden). In order to determine the activity change after exposure to a very alkaline environment, the proteins were solubilized in 0.5 M NaOH. After incubation in NaOH for 24 hours, the samples were run through a NAP-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 30 mM NH_4Ac. The protein containing samples were lyophilized and resolubilised in 1xHBS to a concentration of 800 nM in order to be analyzed in the BIAcore 2000.

BIAcore analysis

A BIAcore 2000 instrument (Biacore AB) was used for real time affinity analysis. HSA and IgG were immobilized on two different surfaces of a CM5 sensor chip by amine coupling to the carboxylated dextran layer. This coupling was done according to the manufacturer's recommendation (Biacore AB). The IgG surface was used as a control. The samples were run through a 0.45 μm filter and injected over the surface in a random order at a flow rate of 5 μl/min. The results clearly show that ABDwt loses the affinity towards HSA during exposure to alkaline solution whereas the mutated variant that lacks four asparagine residues, has the affinity preserved. The sensorgrams are shown in Figure 5.

Also, an analysis of the kinetic parameters was performed for the proteins that still remained active. This was done by analysing the binding behaviour in different concentrations and thereafter calculating the binding constants by using BIAevaluation 2.1 software (BIAcore AB). The concentrations used were 40-220 nM for ABDwt, 200-600 nM for ABDmut and 800-1250 nM for the NaOH treated ABDmut. The results are shown in table 1 below.

Table 1

	K_{on} (1/Ms)	K_{off} (1/s)	K_{aff} (1/M)
ABDwt	9.0×10^4	1.3×10^{-3}	6.9×10^7
ABDmut	1.8×10^4	1.2×10^{-3}	1.5×10^7

K_{on} represents the rate at which the proteins associate, K_{off} the dissociation rate and K_{aff} is the affinity constant which is calculated as the ratio between K_{on} and K_{off}.

Example 4 - Preparation of affinity matrices

In order to evaluate the usability of the stabilized HSA-binding protein in biotechnology, two affinity chromatography matrices were prepared; one with the unmutated protein (ABDwt) as a control and the other with the mutated ABD (ABDmut). The matrix used was Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) with carbodiimide CMC (Sigma Aldrich, Sweden) as the coupling reagent. ABDwt (4.5 mg) and ABDmut (8.5 mg) were dissolved in 9 ml water separately. 3.5 and 5.0 ml gel was added to ABDwt and ABDmut solutions respectively. Finally 0.38 g of CMC was added to the solutions and they were incubated at room temperature over night. The gels were packed on HR columns (d=5 mm, h=43 mm) and pulsed with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 in order to inactivate the matrix and 0.1 M NaAc, 0.5 M NaCl, pH 4 in order to wash unbounded proteins out. After this, the columns were ready to use for affinity purification of HSA.

Example 5 - Retained selectivity

To investigate if the selectivity of the mutated protein towards HSA was retained after exchanging four amino acids, a capture experiment was done. A culture of *E. coli* cells grown over night was fractured by sonication, centrifuged at 40,000xg and filtered through a 0.45 μm filter. The soluble fraction of the *E. coli* lysate was mixed with pure HSA and the mixture was applied on the ABDmut affinity column. After washing the column, the bound material was eluted by lowering the pH to 2.8 as described by Stahl et al., (1989) supra. As can be seen in Figure 4, the selectivity is retained in the mutated variant of ABD.

Example 6 - Alkaline resistant affinity columns

In order to explore the difference between the ABDwt column and the ABDmut column in respect of stability against alkaline treatment, both columns were repeatedly washed with 0.5 M NaOH. By using the AKTA explorer the columns were loaded with HSA, the protein was eluted by lowering the pH (Ståhl et al., (1989) supra) and finally both columns were washed with NaOH. This cycle was repeated 15 times and the total time of exposure exceeded 6 hours. The flow rate used was 60 cm/h and the eluted material was collected and analyzed in each round. In Figure 6 the decrease in capacity is plotted against the NaOH exposure time. As can be seen in the figure, ABDwt is losing the activity quite fast while the mutated variant is keeping the activity throughout the experiment. These results corroborate with the BIACore data, the mutated variant is much more stable against cleaning-in-place (CIP) treatment than the wild type molecule.

Example 7 - Production and purification of Z-ABDstab

A frozen E. coli RRDM15 culture, harbouring the
expression vector pTRPZABDstabT1T2 was used to inoculate
20 ml Tryptic Soy Broth (30 g/l) (Difco, Detroit, MI,
USA) supplemented with 5 g/l yeast extract (Difco) and
50 mg/l kanamycin monsulphate (Labkemi, Stockholm,
Sweden). 10 ml of the over night culture was used to
inoculate 500 ml of fresh medium. The cells were
allowed to grow at 37°C and expression of the proteins
was induced at mid-log phase ($A_{600nm}=1$) by adding 3-β-
indole acrylic acid (SIGMA-Aldrich, Stockholm, Sweden)
to a final concentration of 25 mg/ml. After 24 hours
the cells were harvested by centrifugation at
approximately 5000g for 10 minutes followed by
resuspension in TST buffer (25 mM Tris-HCl pH 7.5, 150
mM NaCl, 1.25 mM EDTA, 0.05% Tween20). The cells were
disintegrated by sonication (Vibracell, Sonics &
Materials, Danbury, CT, USA) and centrifuged at 30000g
for 20 minutes. The supernatant was filtered through
0.45 μm filters (Millipore Corp., Bedford, MA, USA) and
subjected to HSA affinity chromatography (Nygren et al.
1988). The amount of eluted protein was estimated by
absorbance measurements using the specific absorption
constant, a (L/g*cm), and relevant fractions were
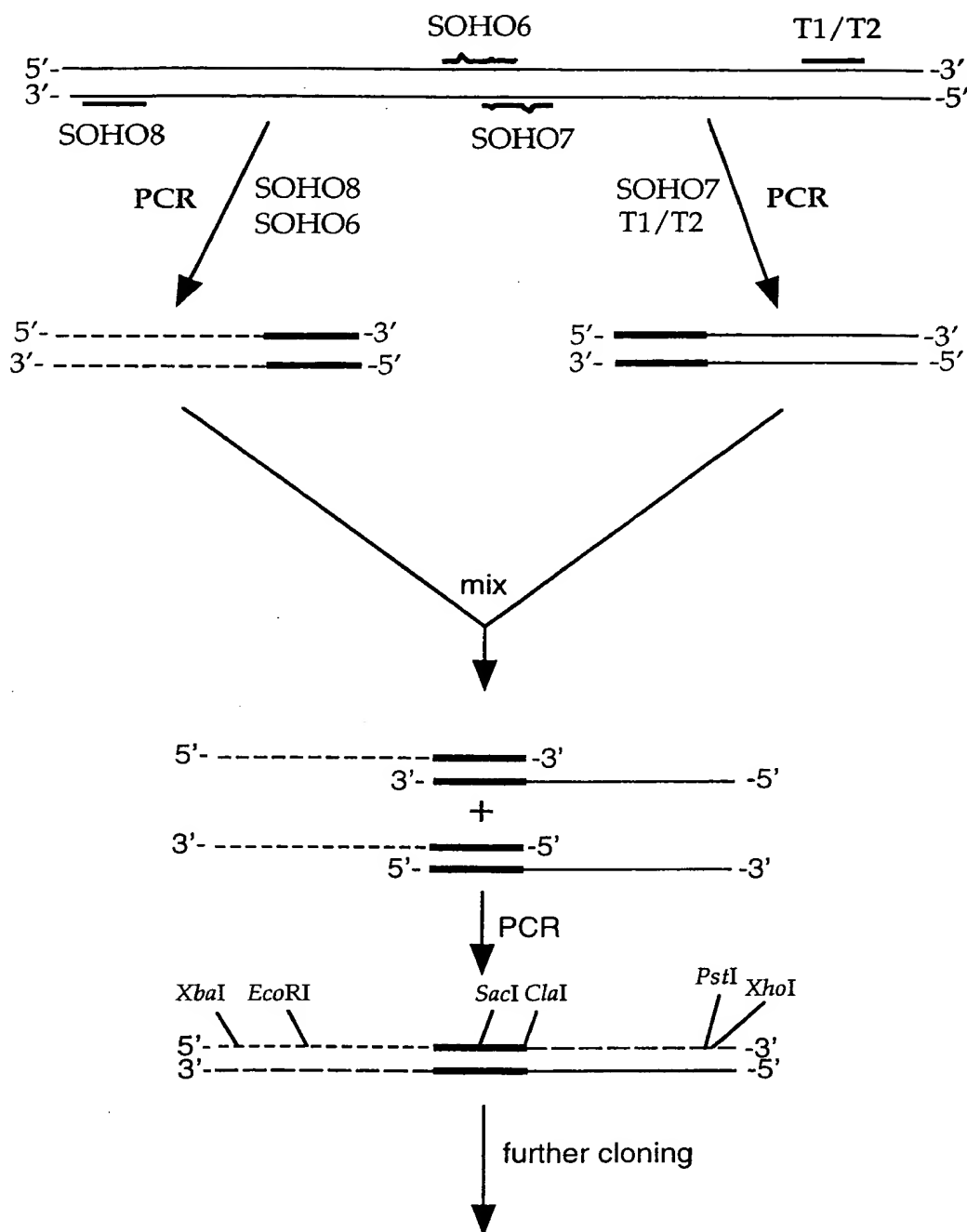lyophilized.

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Fig. 1

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fig 2

from PCR

PstI XhoI
3'
5'

SacI ClaI

XbaI EcoRI

XbaI
5'
3'

Cleavage
XbaI
PstI

pTrpABDT1T2

PstI XhoI
HindIII

XbaI FspI EcoRI

E' ABD

T1T2

P_trp

Cleavage
XbaI
PstI

Ligation

SacI ClaI PstI XhoI
HindIII

XbaI FspI EcoRI

E' ABD

T1T2

P_trp

Transformation

Control cleavage of the plasmids with
SacI och ClaI prior sequencing

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MKAIFVLNAQHDEAVDANS LAEAKVLANRELDKYGV-SDYYKNLINNAKTVEGVKALIDEILAALP

leader → linker → SPG B2 → SPG A3 → SPG S →

1   10   20   30   40   50   60

L   D   DK

ABDwt

ABDmut
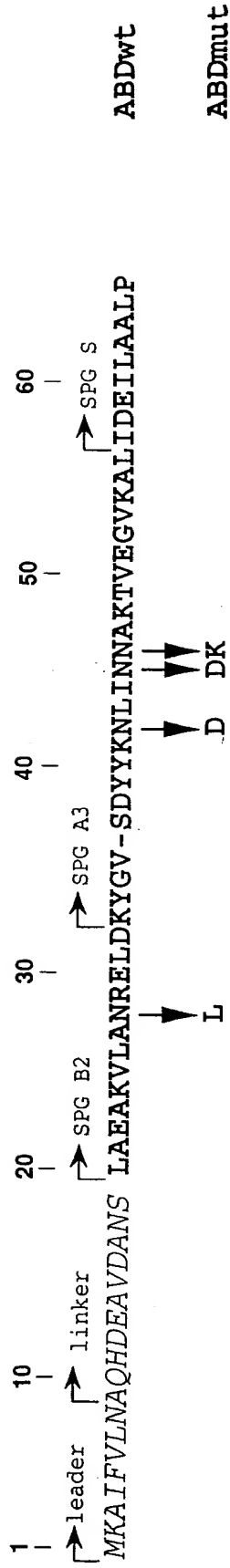
fig. 3

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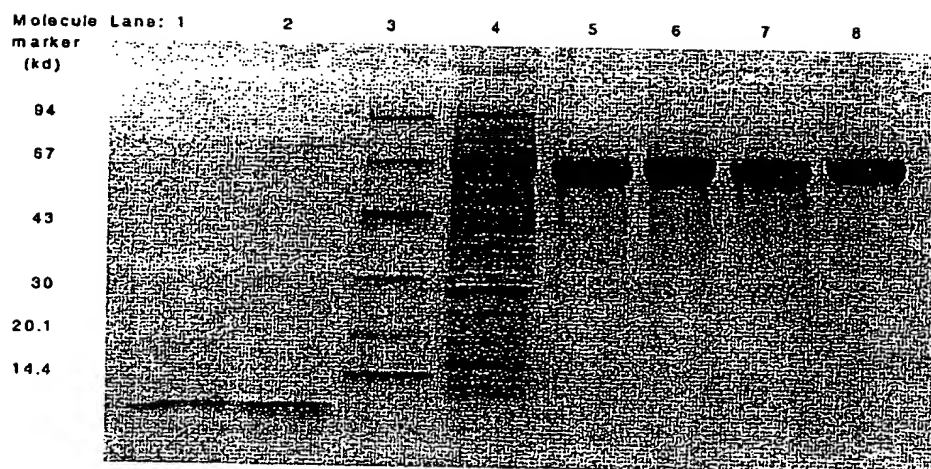
Fig. 4

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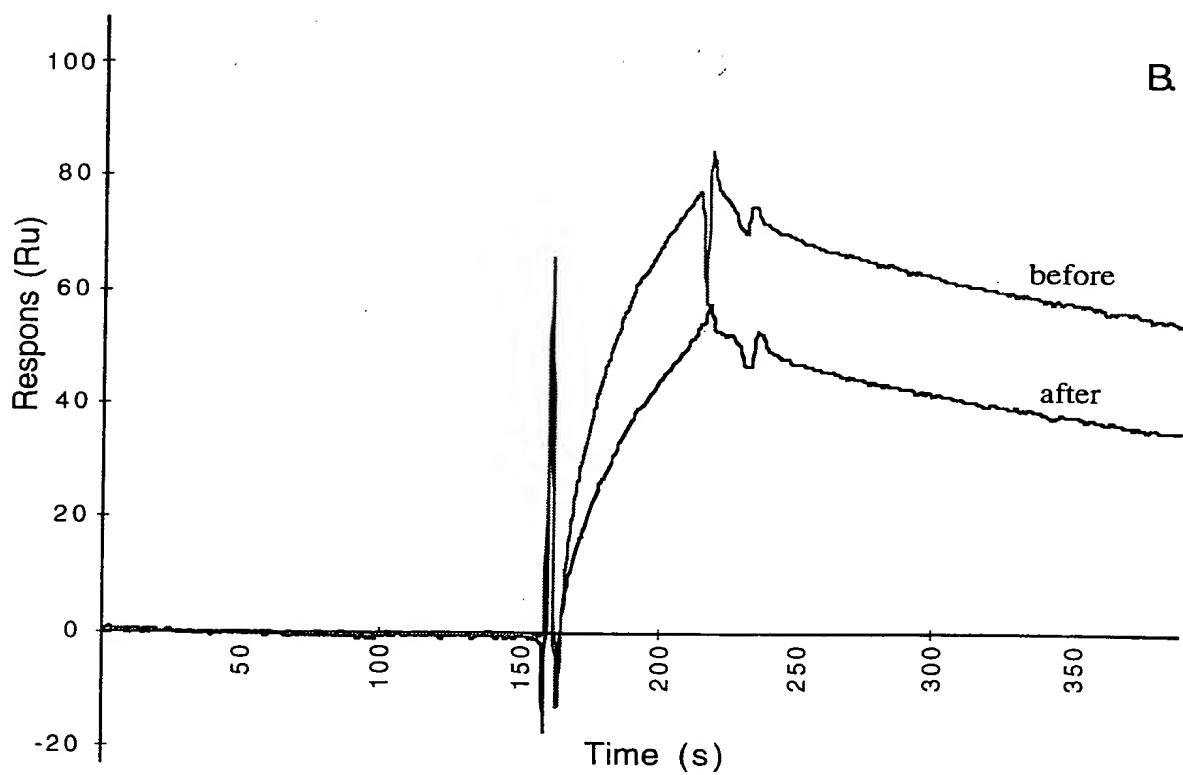
Fig. 5

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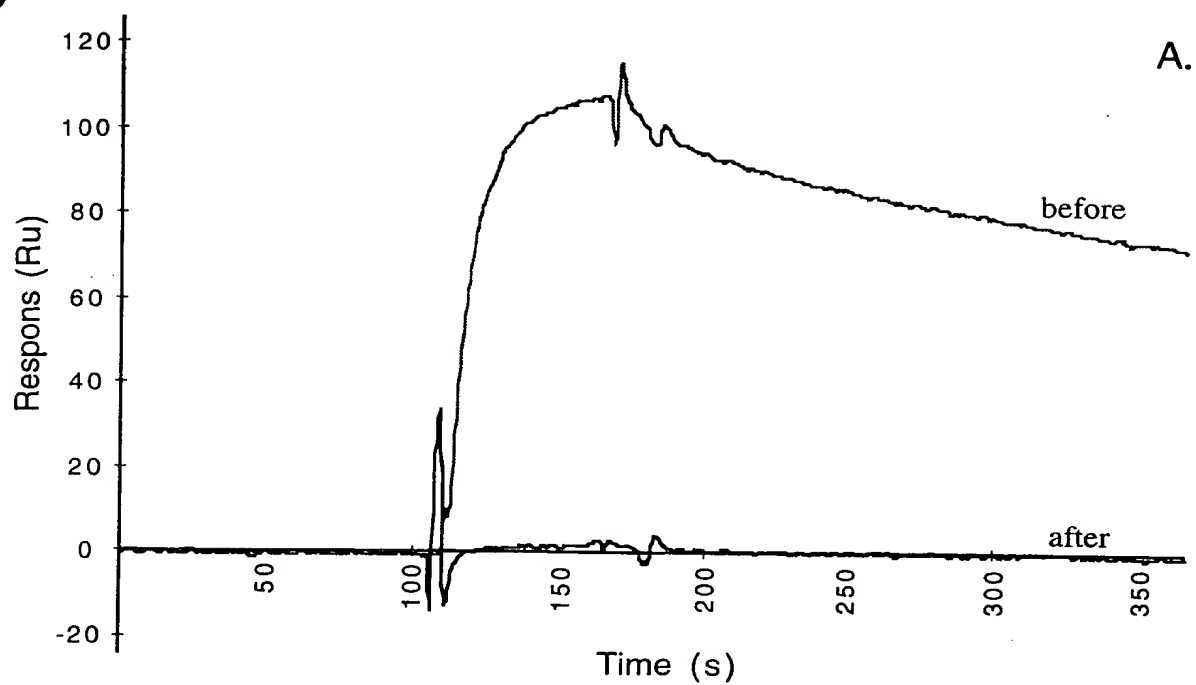fig. 6
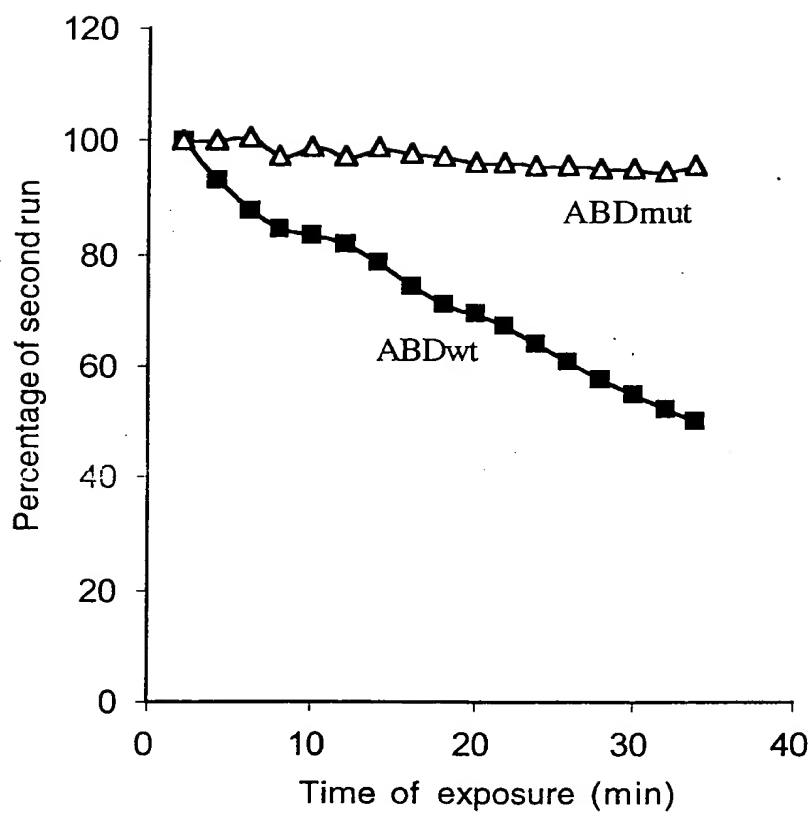
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Frook J. Dehn + Co